Drs. Bing
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Miscellaneous #912

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

Date: May 16, 1973

1. Name of Investigator(s): (include Title and Degrees)

Samuel G. McClugage, Jr., Ph.D., Assistant Professor, Department of Anatomy
Marilyn L. Zimny, Ph.D., Professor, Department of Anatomy

2. Institution &

Address: Louisiana State University Medical Center
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3. Short Title of Project:

Microvascular Response of Fetus to Carbon Monoxide or Nicotine

4. Proposed Starting Date:

October 1, 1973

5. Anticipated Duration of this Specific Study:

October 1973 through September 1976

6. Brief Descripton of Objectives or Specific Aims:

(PLEASE SEE PAGE 2)

7. Give a Brief Statement of your Working Hypothesis:

(SEE PAGE 3)

6. Brief Description of Objectives or Specific Aims:

There are increasing numbers of reports in the literature which suggest that smoking during pregnancy can cause alterations in the normal development of the fetus in utero. Some of the alterations described are decreased neonatal birth weights, greater incidence of premature delivery, increased incidence of spontaneous abortion, and a higher incidence of stillbirths or neonatal deaths of children born from mothers who smoke. Other authors disagree with the causal relationship between impairment of fetal development and maternal cigarette smoking since they believe that social class, background, and other environmental factors which may affect the mother can have just as profound an effect on the fetus as maternal smoking per se. In the past, many have felt that the nicotine content of cigarette smoke may be the etiologic agent causing alterations in the fetus by possibly crossing the placental barrier. Since nicotine has been demonstrated to have so many pharmacological effects on animals and even man, it was only natural to strongly suspect that it was the harmful agent in cigarette smoke. However, when one examines a list of compounds which have been isolated in the gaseous phase of cigarette smoke, he can readily identify other substances which may too have an effect on a growing fetus in utero. One such compound is carbon monoxide (CO). Cigarette smoke is known to have a relatively high content of CO which in living animals competes with oxygen for binding by hemoglobin. This binding of CO by hemoglobin forms an inactive pigment called carboxyhemoglobin (COHb) which causes a proportional decrease of the oxygen carrying capacity of the blood by shifting the oxyhemoglobin dissociation curve to the left (decreases the unloading tension of oxygen). Since CO is known to cross the placental barrier in various

animals and man, it may be responsible for the reported effects of maternal smoking on fetal development which heretofore may have been attributed to nicotine. Thus, using an in vivo microscopic method, a study will be conducted in rabbits to observe the responses of the fetal microvascular system after the maternal exposure of carbon monoxide in one group and after the exogenous administration of nicotine to the mother in a second group. The fetal microvascular response in these two experimental groups will then be compared to that of the mother. Exactly how the maternal exposure to cigarette smoke containing the CO and nicotine causes the reported alterations in the fetus has not been adequately described due, in part, to the difficulty in studying in vivo the fetus while maintaining homeostasis. After in vivo observations, tissue samples from the microvessels will be taken in order to prepare them for transmission or scanning electron microscopy. Thus, the in vivo observations can be correlated with tissue sections selected for study by scanning or transmission electron microscopy. This study is designed to specifically examine in vivo the separate effects of nicotine or CO on the fetal microvascular system in an attempt to provide further information on the reported harmful effects of cigarette smoke on the unborn and even adults; thus, the adverse effects of maternal smoking on fetal development and its reported etiologic role in the development of cardiovascular diseases in adults may be better understood.

7. Brief Statement of Working Hypothesis:

Due to previous work done in my laboratory and work done by others, CO may induce alterations in the fetal or adult microvascular system which seriously compromise blood flow to tissues or organs. This reduction of blood flow would seriously reduce the oxygenation of fetal or adult tissues; this, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of

COHb. Nicotine has not yet been studied in this regard. In vivo microscopy offers the possibility of measuring vascular dimensions and at the same time observing any changes in the behavior of blood in the microvessels. It is quite conceivable that carbon monoxide and/or nicotine may both alter the dynamic structure and function of the fetal or adult microvascular system which, in turn, will reduce the proper delivery of blood to tissues or organs.

8. Experimental Design and Significance:

A. Experimental Design:

In one experimental group, the mesenteries of fetal and adult pregnant rabbits (New Zealand albino) will be studied. The pregnant rabbits will be anesthetized with Urethane I.V. (ethyl carbamate) or with methoxyflurane using a closed circuit anesthetic machine. To study the fetal circulation, a fetus is exteriorized from the uterus of the mother leaving the placental circulation and fetal membranes intact on various days of gestation between days 25 and 32 and the fetal mesentery is exposed surgically. Homeostasis is maintained by irrigating the field of study with Ringer's solution warmed to the body temperature of the animal by regulating heaters. Gauze sponges covering the fetus and surrounding the mesentery provide insulation for the animal during the experiment. Furthermore, ambient air surrounding the fetus is maintained at bodily temperature (37.5°C) using a Sage air incubator. To study the mesentery of the adult animal, the bowel is displaced after laparotomy and a loop of bowel is exposed. Homeostasis is maintained as in the fetus.

To study the exposed mesentery of the fetus or adult pregnant animal, a beam of monochromatic or white light is brought to the undersurface of the mesentery via a hollow, fused quartz rod; subsequently, the mesentery is transilluminated and examined with a Leitz stereo binocular microscope equipped with 2.5X, 4X, and 10X objectives and 10X and 16X oculars. Measurements of the microvasculature within the mesentery will be performed by a Leitz eyepiece micrometer. Alternatively, the optical images from the microscope will be projected onto the photocathode of a Cohu, RCA, or Fairchild/Dumont vidicon television system and kinerecorded with a Bolex H-16 Rex 5 16mm. motion picture

camera. The use of monochromatic light used in conjunction with a black and white television system greatly improves the visualization of living tissues or organs since the contrast is greatly increased. The 16 mm. motion picture camera may also be used for direct cinéphotomicrography. Throughout the in vivo experiments, the results are permanently documented for later reference. These results can be studied repeatedly and critically analyzed frame by frame in order to compare the sequential responses of the microvasculature in one animal to that of other animals. Thus, using this in vivo technique, the rate, duration, magnitude and direction of the response in the fetus or adult animal can be examined and recorded.

In one group of experimental animals, studies will be continued on the response of the fetal microvascular system to the maternal exposure of CO. To study the acute response of the fetal microvasculature to CO, the mother will receive a mixture of CO and air in varying concentrations of CO from .01% to .1% balance/air (100-1000 p.p.m.) using a closed circuit anesthetic machine. This range of CO will cause an increase in the COHb level of the female adult rabbit from 5 - 15% which mimicks COHb levels which have been reported in human studies on mothers who smoke. The fetal microcirculation will then be studied under the following experimental conditions: (1) after maternal anesthesia but before CO exposure, i.e., while the mother is breathing room air or air via the closed circuit anesthetic machine; (2) during maternal CO exposure; (3) during recovery when the mother is again allowed to breath room air. It should be emphasized that each pregnant animal can be used for each of the above experimental (CO) groups. Thus, each animal can be used as its own "control". The response of the fetal microvascular

system to CO will be compared to that of the adult microvascular system in order to compare the sensitivity of the fetus with that of the mother.

In a second group of experimental animals, the effects of nicotine on the fetal microcirculation will be examined after the administration of a subcutaneous dose to the mother. The dose to be used in rabbits will attempt to mimick that amount of nicotine absorbed by human smokers, which has been reported to be 1.0 to 2.0 mg. of nicotine per kilogram from a pack of cigarettes per day. As in the CO experimental group, each fetal preparation will be examined before, during, and after the maternal administration of nicotine. The response in the fetus will also be compared to that of the adult microvascular system.

After completion of experiments on the effects of CO or nicotine on the fetal microvascular system, the results from the two experiments will be compared for any similarities or differences in responses under the two experimental conditions. A third group of experiments which would be most interesting to perform would be to study the response of the fetal microvascular system during the maternal inhalation of cigarette smoke. However, to date, I am not aware of any mechanical device for exposing rabbits to cigarette smoke under conditions which simulate human exposure. There are, of course, means by which the effects of cigarette smoke could be examined in rabbits, but I question the value of these studies if they do not at least simulate conditions during human smoking. If such a device for rabbits becomes available during the course of the experiments, it could be easily incorporated into the experimental design of this study. I know that the Council for Tobacco Research is sponsoring work to develop mechanical devices for animals which simulate human conditions; thus, they are in a position to know when such a device for use with rabbits becomes available. The <u>in vivo</u> model which is to be used in the

various experimental groups would be a means for examining the effects of maternal smoking on the response of the fetal microvascular system, and then to compare this response with the nicotine-treated and CO-treated animals.

One further experimental group of animals will be used to study in adult female Sprague-Dawley rats (100-125g) the response of the mesenteric and hepatic microvascular system to carbon monoxide or nicotine. Animals will be anesthetized by intraperitoneal injection of urethane (ethyl carbamate). To expose the liver or mesentery of the rat, a midline and subcostal incision will be made and the liver or mesentery exteriorized by floating it onto a window of Saran Wrap which overlays a substage condenser of a Leitz Panphot microscope. Homeostasis will be maintained by irrigating the field with physiological Ringer's solution kept at the body temperature of the animal by heat regulators. Transillumination of the tissue will be accomplished by using a technique modified after Bloch and Coyas (Anat. Rec. 145: 374, 1963). With the liver or mesentery in position over the substage condenser, microscopy of the tissue is accomplished by passing a beam of monochromatic light through the condenser of a modified Leitz Panphot microscope. As mentioned earlier, the use of monochromatic light aids in the visualization of tissues and when used in conjunction with a black and white television system, the contrast can be greatly improved. The transilluminated tissues can then be observed by direct microscopy at magnifications of 100-1200X using Leitz water immersion or U.M.K. objectives with appropriate oculars or the optical image can be projected onto the photocathode of a television system.

The adult rats will be exposed to CO and nicotine in a similar manner as described for rabbits before, during, and after exposure. This experimental

group of animals will provide information on another species of animal which can then be compared to the response of the microvascular system in adult rabbits. Furthermore, the use of a microscope (Leitz Panphot) which permits higher magnifications (100-1200X) than a stereo-binocular microscope may provide information which would not be obtainable with lower magnifications and poorer resolutions. Also, the microvascular system of liver is morphologically and functionally different from that of the mesentery; thus, the sensitivity of the two to CO or nicotine may also be quite different since one represents a microvascular bed in a relatively non-metabolic tissue (mesentery) versus one which is highly metabolic (liver).

During the experiments, the maternal and fetal hematocrit, hemoglobin concentration (g/100ml), oxyhemoglobin concentration (%) and carboxyhemoglobin (%) will be monitored, the latter three by an IL CO-Oximeter, in order to compare the response in the fetal or adult microvascular systems with any fluctuations in maternal or fetal blood parameters.

In each of the various experimental groups, samples of blood vessels will be taken after <u>in vivo</u> observations. These samples of blood vessels will be fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4 for 24 hours, rinsed three times with buffer and stored in a refrigerator. Part of the sample will then be osmicated, dehydrated in graded alcohols, processed through amyl acetate and critical point dried. After drying, the tissue will be coated with carbon and gold palladium alloy and viewed in a JSM-U3 scanning electron microscope. The usual accelerating voltage used by the investigator in past studies of other tissues has been 15 KV. Observations of the tissue in question will be made at this accelerating voltage and other magnitudes of voltage will be tested so as to obtain the maximum visual results.

Following scanning electron microscopic observations, the sample may be placed in propylene oxide and embedded in plastic for further study with the transmission electron microscope. The plastic embedment material used in our laboratory is either Maraglas or Araldite. After polymerization of the plastic embedment, 1 μ plastic sections will be stained with Paragon and viewed with a light microscope for purposes of orientation. Ultra thin sections will then be made, stained with uranyl acetate and lead citrate and viewed with an A.E.I.-6B transmission electron microscope.

In addition to viewing the same piece of tissue with both scanning and transmission electron microscopy, it will also be possible to use part of the original sample that was fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4, solely for transmission electron microscopic observation. For this, part of the original fixed sample would be osmicated, dehydrated in graded alcohols, placed in propylene oxide and subsequently be embedded in Maraglas. Once again thick plastic sections would be stained with Paragon and the following ultra thin sections would be stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope.

If deemed necessary, for correlation with scanning or transmission electron microscopic observations, part of the originally fixed sample can also be prepared for light microscopy. For this purpose part of the original fixed sample would be dehydrated in graded alcohols and embedded in paraffin. Paraffin sections could then be stained for routine histological observations or stained with special chemicals so as to visualize various fibrous components of the tissue or possible lipid inclusions.

B. Significance:

The specific response of the fetal microvascular system to maternal exposure of carbon monoxide or nicotine has not been reported due, in part, to the difficulty in studying these vessels in vivo with the light microscope while maintaining homeostasis. In general, the response of the fetal microvascular system to any maternal agent is poorly understood due, in part, to the lack of information in man and animals regarding the transfer of substances across the placenta to the fetus.

During this past year, my laboratory has been conducting in vivo studies on the response of the fetal microvascular system to maternal carbon monoxide exposure. The main purpose of this study was to observe any changes in the dynamic structure or function of the fetal microvascular system which may occur after exposure of the mother to carbon monoxide in order to possibly explain the reported cause-and-effect relationship between maternal smoking and impairment of fetal growth and development. Since mothers who smoke have increased circulating carboxyhemoblobin (COHb) levels, possibly the carbon monoxide (CO) per se may have detrimental effects upon the fetus, particularly since CO is known to cross the placental barrier.[1] In this regard, Astrup et al.[2] found that mothers exposed to 180 p.p.m. of CO had a 20% decrease in birth weight and a neonatal mortality rate of 35%. They suggested that the CO content of cigarette smoke may be responsible for these two occurences. In studies conducted by Meyer and Comstock,[3] perinatal mortality increased if the mother had smoked during pregnancy. Several authors[4-9] have suggested that the lower birth weights and increased mortality of babies born from mothers who smoke may be related to the relative hypoxemia in the fetus caused by the CO since babies born at high altitudes often have similar

incidences of lower birth weights or mortalities. Many of these reports strongly suggest a cause-and-effect relationship between CO and fetal development in mothers who smoke. The hypoxemia which occurs from exposure to CO is most often mentioned as the harmful effect elicited by CO per se, either from cigarette smoke or other sources. If the hypoxemia is truly responsible for the pre-natal or post-natal alterations from mothers exposed to CO, then the only way this hypoxic effect might be overcome would be by either increased production of hemoglobin by the mother, or an increased maternal blood flow to the placenta.

Experiments conducted in my laboratory during the past year, however, suggest that CO may have other effects upon the fetus in addition to its known effects upon the oxyhemoglobin dissociation curve. My experiments to date have shown that carbon monoxide administered to the mother at concentrations of 100-1000 p.p.m. in an air mixture will cause an increase in the maternal and fetal COHb level in rabbits comparable to or slightly higher than that of smokers (5-25% COHb). The exposure to CO in the mother causes a linear increase in her COHb levels throughout a four hour observation period. Fetal blood samples taken after completion of in vivo observations on the fetal mesentery revealed a similar or slightly higher per cent of saturation of hemoglobin by carbon monoxide. The oxyhemoglobin level (%) decreased in the adult concomitant with the rise in COHb levels. The hematocrit and total hemoglobin (g/100ml) did not change appreciably throughout the course of the experiments.

The response of the fetal microvascular system to increased levels of COHb is a vasoconstriction in the small arteries and veins (100-300μ I.D.) of the mesentery followed by a progressive decrease in the linear velocity of blood flow through these vessels. These hemodynamic events preceded the eventual breakdown of the endothelial lining of the capillaries and

post-capillary venules resulting in the formation of petichial hemorrhages along the course of these vessels (capillaries and post-capillary venules) and widespread congestion within the capillary network of the fetus. The per cent of vasoconstriction (compared with control, before CO administration) in the fetus increased with time and with the level of maternal COHb. The observation period was never longer than four hours. Furthermore, the degree of extravasation of red blood cells from the capillaries or post-capillary venules and the amount of congestion within the microscopic field also increased with time and with the level of maternal COHb. The maximal response to the increased COHb levels was cessation of flow through terminal arterioles, capillaries, and post-capillary venules, with a great reduction in the linear velocity of blood flowing through the small arteries and veins in the mesentery. Due to the congestion within the capillary bed, the majority of the blood flowing in the small arteries would bypass the capillary network by flowing into arteriovenous anastamoses into small veins or venules. Control animals allowed to breath room air or an air/gas mixture did not develop the microvascular lesions observed in fetuses exposed to CO for similar periods of time up to four hours.

Once a high level of maternal COHb was reached, the toxic effects of CO on the fetus were not reversible since removal of the CO stimulus after the initial vasoconstriction does not reverse the further effects upon endothelial permeability of capillaries and post-capillary venules. This is probably explained by the fact that the COHb levels, once elevated (20-25%), will not fall in time to prevent further damage to the endothelium of the capillaries and post-capillary venules. The results suggest, however, that if the CO stimulus is removed before the COHb levels reach 10% in the mother, only a slight vasoconstriction of small arteries and veins will be observed.

It is interesting to compare the results of these experiments with those experiments performed in the past on the response of the fetal mesenteric microvascular system to maternal hypoxia[10]. In this earlier study, maternal hypoxia induced a vasoconstriction in the fetal microvascular bed which seemed to be mediated by an oxygen dependent alpha-adrenergic mechanism since recovery from this vasoconstriction coincided with the return of the pO_2 to normal values after removal of the hypoxic stimulus to the mother. In these experiments, this recovery occurred within 20 minutes.

The results of my experiments suggest that the vasoconstriction in the fetal microvascular system may be due to fetal hypoxemia which occurs with increased levels of COHb since hypoxia alone, induced by a low oxygen gas mixture, produced a similar vasoconstriction. Other authors[11] have also reported increased fetal systemic vascular resistance during hypoxia in pregnant ewes, although one cannot assume that an increased fetal systemic vascular resistance reflects what is occuring in a particular microvascular bed of an animal.

It is more difficult to explain the endothelial damage induced by CO per se or hypoxia. In this regard, the extravasation of red blood cells through the endothelium represents some type of endothelial damage. This increased permeability of endothelium after exposure to CO has also been described by other authors in "adult" animals or human studies. Astrup[12] found that cholesterol-fed adult rabbits exposed to CO had a greater accumulation of cholesterol in their arterial walls (aorta) when compared to only cholesterol-fed controls. Furthermore, Astrup and his associates found that CO (9-10% COHb) alone induces arterial lesions hallmarked by subendothelial edema indistinguishable from the intimal appearance of spontaneous arteriosclerosis. Even though the lesions

described by these authors were found in large vessels and only non-cellular plasma constituents permeated the endothelium, their studies still support a direct toxic effect of CO on vascular endothelium. Astrup et al.[13] further described an acceleration by CO alone in the development of atheromatosis of the aorta. They believed that the edematous condition and higher protein content of the aortic walls was due to an increased endothelial permeability. In the ultrastructural studies conducted by Kjeldsen et al.[14] on the intimal changes in the rabbit aorta after moderate CO exposure, edema was evident under the basement membrane as well as the endothelial cells; often endothelium completely separated from the basement membrane and a plague was formed. Of most interest in this study was the presence of tiny hemorrhages with platelet and red blood cell plugging in the areas of denuded endothelium. Kjeldsen et al.[14] concluded that the morphologic intimal changes of the rabbit aorta were due to CO per se since the oxygen tension did not change during CO exposure. This was not the case in my experiments, since oxygen tension did decrease with a concomitant rise in COHb. Siggaard-Andersen et al.[15] also reported that CO induces endothelial damage and that CO has a more pronounced effect than hypoxia alone on the permeability of capillaries to albumin. The exact mechanism by which CO increases endothelial permeability to plasma and/or cellular components of blood remains obscure; however, oxygen dependent enzymes may be necessary in order to maintain the permeability of individual endothelial cells and/or intercellular endothelial junctions. CO may in some way have a direct toxic effect upon these same enzymes.

The results of experiments on rabbit fetuses in my laboratory and those conducted by others on adult animals strongly suggest that CO can compromise the blood flow to tissues by causing a vasoconstriction of

small arteries and veins and by increasing the endothelial permeability to plasma and/or cellular constituents of blood. These functional or morphological alterations can severely compromise the perfusion of capillaries thus impairing the proper delivery of oxygen to tissues or organs. This, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of the inactive pigment, carboxyhemoglobin.

The results from these studies lend further support to the possibility that the CO content of cigarette smoke may be the causative agent which is responsible for the lower birth weights of newborn or the higher incidence of neonatal mortality in newborns from mothers who smoke. The microvascular effects described in this study coupled with the known effects of CO on oxygenation could impair the proper delivery of blood to the growing fetus. The functional and morphologic alterations which may arise in the fetus, then, really only depends upon whether or not the microvascular response observed in the fetal mesentery is truly representative of what occurs in other microvascular beds such as the central nervous system.

The experimental protocol in this proposal will further investigate the effects of CO on the fetal microvascular system. The use of monochromatic light will provide additional information on the alterations in structure and function of the microvascular system induced by CO by enabling more critical observations of the microvascular response at one wavelength of light, for example, 414 mμ for hemoglobin. Since studies will also be conducted on the maternal microvascular response to CO, it will be interesting to ascertain if this response mimicks that in the fetus. Since several of the ultrastructural studies by Kjeldsen et al.[14] and others suggest that a

mild exposure to CO can induce pathologic changes in the walls of vessels in rabbits, it is conceivable that these changes could have an effect upon the flow of blood through these vessels. Thus, the experiments on the response of the adult microvascular system will be compared to: (1) the response of the fetus; (2) ultrastructural studies conducted by other authors in adult animals or man; and (3) the ultrastructural results of our own studies. If CO increases the permeability of endothelium to plasma constituents, then it may well be that CO does play a significant role in the development of coronary heart disease and even peripheral vascular diseases as has been suggested by several authors. If our scanning and transmission electron microscopic studies of fetal blood vessels which have been exposed to CO demonstrate a structural similarity to adult blood vessels that have been exposed to CO, then can one conclude that such morphologic changes might predispose a newborn animal (or human) to cardiovascular disease in later life? The dovetailing of information gathered from vital microscopic, scanning electron microscopic, and transmission electron microscopic studies of fetal vessels exposed to CO in this study may either support or refute this possibility.

The results form the CO experimental groups will be compared to the results from the nicotine experimental groups. Little information is available on the effects of nicotine on the fetus. The potentially harmful effects of nicotine on the fetus are just as important as those effects which may be related to CO. In fact, nicotine has been implicated more often than CO as the main harmful constituent of tobacco smoke due, in part, to its known pharmacological effects on the cardiovascular system. Nicotine is known to cross the placental barrier in some animals such as rats.[16] In these animals, the fetal levels of nicotine actually exceeded the maternal levels at various intervals of time after maternal administration of radioactive nicotine. Nicotine is known to induce a significant

increase in the amount of catecholamines released from the adrenal gland.[17] If nicotine does cross the placental barrier in pregnant rabbits and if it does increase circulating levels of catecholamines due to its action on the fetal adrenal gland, then the peripheral vascular resistance and/or cardiac output might be markedly affected. Nicotine is also known to have other effects such as accelerating platelet aggregation by ADP.[18] Like CO, nicotine also has been shown to affect the birth weight and neonatal or prenatal mortality rate of offspring of mothers who received nicotine.[19] The vasoconstriction and breakdown of the endothelial lining of the fetal microvascular system after CO may also occur with nicotine since Matsubara and Sano[20] suggested that nicotine induces closure of pre-capillary sphincters in calves causing a decreased capillary filtration coefficient. Although the studies by Matsubara and Sano[20] were performed in calves, other authors have described the effects of nicotine on the fetus and have suggested that the response depends upon the gestational age of the fetus which, in turn, reflects the development of the autonomic nervous system and adrenal gland. Thus, nicotine can play a similar role as CO in compromising the blood flow and/or oxygenation of growing fetuses; thus it is put in a similar category as a potentially harmful etiologic agent of tobacco smoke. It will be interesting to compare the response of the fetal microvascular system to the exogeneous administration of nicotine to the mother with the response of maternal carbon monoxide esposure. Then one may be able to better appreciate the mechanisms which function in the fetus to produce deleterious effects upon fetal growth and development in mothers who smoke.

As mentioned in the experimental protocol, the studies conducted on the response of the "adult" microvascular system in rabbits after exposure to CO or nicotine will be repeated in adult rats. Thus, <u>in vivo</u> observations

will be conducted on the response of the mesenteric microvascular system in rats to CO or nicotine and compared to the response in "adult" rabbits. This will permit a comparison of the sensitivities to CO or nicotine of these two animals in the same microvascular bed. Furthermore, in order to study the effects of CO or nicotine on another microvascular bed, in vivo microscopic studies will be conducted in rats on the response of the hepatic microvascular system to CO or nicotine; these results will then be compared to the response in the mesentery. This will provide useful information on the sensitivities of different microvascular beds in the same animal (hepatic and mesenteric in rats) versus similar microvascular beds in two different animals (mesenteric in adult rabbits and rats).

After completion of the acute experiments outlined in this proposal, hopefully additional information will be available which either supports or refutes (a) the reported cause-and-effect relationship between cigarette smoking and fetal or neonatal development, and (b) the etiologic role cigarette smoking plays in the development of various cardiovascular diseases in adults. By examining the separate effects of CO and nicotine on the adult and fetal microvascular systems in animals, one may gain a better insight into the problem of defining what constituents of cigarette smoke are truly harmful.

C. Addendum:

In the experimental protocol, one potential experimental group was the effects of maternal smoking on the fetal or adult microvascular system. These studies depended upon the availability of a mechanical device which would simulate human exposure to cigarette smoke. I would like to re-emphasize that if such a machine becomes available, this experimental group (exposure to smoke) will be added. I believe that this would be an integral part of the proposal since one could observe in the living animal if the CO -

response or the nicotine-response more closely followed the microvascular response of exposure to cigarette smoke.

D. References:

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9. Physical Facilities Available :

The senior investigator has 400 square feet of laboratory space equipped with the following items.: 1 vibrationless steel optical bench for vital microscopy; quartz-rod apparatus; Leitz stereo binocular microscope modified for vital microscopy; Leitz Panphot microscope (without optics); RCA Vidicon television system; Fairchild/Dumont Vidicon television system; 8" Conrac TV monitor; Bolex H-16 Rex 5 16 mm. motion picture camera adapted for cinémicrophotography; 2-tripods; motion picture editing and storage equipment; YSI temperature control equipment; balances; Sage air curtain incubator; Heidbrink closed-circuit anesthetic machine; Wilmot Castle surgical lamp; Bausch and Lomb spectrophotometer; A. O. microtome; warming table; clinical centrifuge; A. O. microstar microscope; deionizer; microhematocrit centrifuge; IL 182 CO-oximeter.

Dr. Zimny has in her laboratory a A.E.I. - 6B transmission electron microscope; furthermore, she has available for her use a scanning electron microscope, J.S.M. - U3, at Touro Infirmary in New Orleans.

The Department of Anatomy also maintains adequate dark room and animal care facilities.

10. Additional Requirements:

None

11. & 12. Biographical Sketches of Professional Personnel and Pertinent Publications:

Name: Samuel G. McClugage, Jr.

Birth Date: REDACTED
Birth Place:

Education: Undergraduate: Millikin University, Decatur, Illinois
A.B. (Zoology), 1966.

Graduate: University of Cincinnati, College of Medicine, Cincinnati, Ohio
Ph.D. (Anatomy), 1970.

Honors: N.I.H. Predoctoral Fellowship, 1967-1970

Consultant, Proctor and Gamble Company, Cincinnati, Ohio
1972 -

Recipient, Microcirculatory Society-Pharmacia Travel Award (to visit research laboratories in Scandanavia), June, 1973.

Socities:

REDACTED REDACTED

Research Interests: In vivo microscopy of living cells, tissues, and organs in situ under normal or pathologic conditions; in vivo physiologic and pharmacologic studies; microcirculation; hematology; application of television and electronic techniques to microscopic study of living tissues and organs in situ.

Background:

1. Assistant Professor of Anatomy, Louisiana State University Medical Center, 1971 - present
2. Postdoctoral Fellow in Anatomy, University of Cincinnati, 1970-1971
3. Pre-doctoral Fellow, National Institutes of Health (GM-38179), University of Cincinnati, 1967-1970
4. Pre-doctoral Fellow, from the Dean of the College of Medicine, University of Cincinnati, 1966 - 1967
5. Assistant Instructor in Biology, Millikin University, 1966

Publications:

1. McCuskey, R. S., McClugage, S. G., Moore, T. J., and Miller, M. L. Response of the fetal mesenteric microvascular system to maternal hypoxia. Proc. Soc. Exp. Biol. & Med. 132: 636-639, 1969.

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4. McClugage, S. G., and McCuskey, R. S. Relationship of the microvascular system to bone resorption and growth in situ. Microvas. Res. In press.

5. McClugage, S. G., and McCuskey, R. S. Microscopic study of the response of the living liver to carbon tetrachloride poisoning. Microvas. Res. 5: 354-360, 1971.

6. McCuskey, R. S., McClugage, S. G., and Meineke, H. A. Microscopy of living bone marrow in situ. Experimental Hematology 21: 33-34, 1971.

7. McClugage, S. G. Response of the fetal microvascular system to maternal carbon monoxide exposure (In preparation).

NAME: Marilyn L. Zimny

TITLE: Professor of Anatomy

BIRTHDATE: REDACTED

PLACE OF BIRTH: REDACTED

EDUCATION:

University of Illinois, Urbana, Illinois
Chemistry - major, Zoology - minor, B.A., 1948
Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, M.S., 1951
Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, Ph.D., 1954

PROFESSIONAL EXPERIENCE:

Professor - Anatomy, Louisiana State University Medical Center, 1964 - present
Associate Professor - Louisiana State University Medical Center, 1959 - 1964
Assistant Professor - Louisiana State University Medical Center, 1954 - 1959
Visiting Professor in Anatomy, University of Costa Rica, School of Medicine, February-June, 1961 - 1962
Sabbatical leave, Institute of Arctic Biology, University of Alaska, 1966
Abstractor for Biological Abstracts, 1959 to present
The World Book Encyclopedia Biology Committee

ORGANIZATIONS:

REDACTED

REDACTED

PUBLICATIONS:

1. Zimny, M. L. and Rigamer, E. Glomerular ultrastructure in the kidney of a hibernating animal. Anat. Rec. 154: 87-94, 1966.

2. Zimny, M. L., Sherman, M. and Romano, C. C. Ultrastructural modifications of the intercalated disc during hypothermia in the rat and the ground squirrel. Cryobiology 4: 317-328, 1968.

3. Zimny, M. L. Glomerular ultrastructure in kidneys from some northern mammals. Comp. Physiol. & Biochem. 27: 859-864, 1968.

4. Zimny, M. L. and Redler, I. An ultrastructural study of patellar chondromalacia in humans. Journal of Bone and Joint Surgery 51A: 1178-1190, 1969.

5. Redler, I. and Zimny, M. L. Scanning electron microscopy of normal and abnormal articular cartilage and synovia. Journal of Bone and Joint Surgery 52A: 1395-1404, 1970.

6. Zimny, M. L. and Redler, I. An ultrastructural study of chondromalacia fabellae. Clinical Orthopaedics and Related Research 82: 37-44, 1972.

7. Zimny, M. L. and Redler, I. Scanning electron microscopy of chondrocytes. Acta Anat. 83: 398-402, 1972.

8. Booth, W. V., Zimny, M. L., Kaufman, H. J. and Cohn, I. Scanning electron microscopy of small bowel strangulation obstruction. Amer. J. Surg. 125: 129-133, 1973

9. Zimny, M. L. and Redler, I. Variations in morphology of cartilage within a given area of articular surface. (Submitted for publication, J. Microscopy).

13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Samuel G. McClugage, Jr.	50%	
Marilyn L. Zinny	15%	REDACTED
Technical		REDACTED
*Research Assistant (including fringe benefits)	100%	
*Research Assistant (including fringe benefits)	50%	
Secretary (including fringe benefits)	50%	
Sub-Total		REDACTED
B. Consumable Supplies (list by categories)		
100 Pregnant rabbits @$20.00		2,000.00
25 Non-pregnant rabbits @$10.00		250.00
Anesthetic gases (CO/Air, O_2)		400.00
Motion Picture film and film processing		1,000.00
Misc. supplies (chemicals, surgical instruments, etc.)		600.00
Sub-Total		4,250.00
C. Other Expenses (itemize)		
Animal Care (.20/day/rabbit)		750.00
*100 hours of use of Scanning Electron Macroscope @$20.00/hr.		2,000.00
Travel (for two people to attend one meeting per year)		600.00
*Machinist expenses		400.00
Sub-Total		3,750.00
D. Permanent Equipment (itemize)		
*Monochromatic system adapted for quartz rod		2,150.00
*Optical equipment necessary to adapt Leitz Panphot Microscope for in vivo microscopy		4,790.00
*Low light level Cohu television camera including sync. generator		4,750.00
Sub-total		11,690.00
E. Overhead (15% of A+B+C)	Overhead	3,444.00
	Total	REDACTED

Estimated Future Requirements:

	Salaries	Consumable Supplies	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	4,250.00	3,350.00	1,400.00	3,504.00	28,262.00
Year 3		4,250.00	3,350.00	900.00	3,601.00	28,506.00

Salaries include increments of 6% per year plus 10% for fringe benefits

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

* (See Justification of Budget on next page)

Signature [redacted]
Director of Project Samuel G. McClugage, Jr.
(504) 947-9961 - ext. 255 Telephone

Signature
Business Officer of the Institution E. F. Pohlig
Comptroller (504) 527-5142 Telephone

10035423O5

13. Justification of Budget:

A. Personnel

The personnel that will be required on this project are one full-time research assistant for Dr. McClugage and a half-time research assistant who will work with Dr. Zimny in the preparation of tissues for scanning and transmission electron microscopy. The percent used for calculating fringe benefits at Louisiana State University is 10% which has been included in the "amount column" of the budget.

B. Use of Scanning Electron Microscopy:

The Department of Anatomy at Louisiana State University Medical Center does not have a scanning electron microscope (SEM). However, we have an agreement with the Research Institute at Touro Infirmary in New Orleans to rent their SEM at a rate of $20.00/hour. Dr. Zimny has access to this microscope whenever its use is needed.

C. Machinist Expenses:

The employment of a machinist who can make the necessary animal trays for use on the Panphot microscope, adapted for vital microscopy, will be necessary. These trays must meet certain specifications depending on the type of animal used and the particular organ which is to be observed in vivo.

D. Permanent Equipment :

1. Monochromatic System Adapted for Quartz Rod

Schoeffel Instrument Corporation recently manufactured a monochromatic system which provides maximum light energy (from 200-700nm.) with high spectral purity. The complete system consists of a Xenon or Xenon-Mercury arc lamp, power supply, arc lamp housing, double monochromators, and appropriate optics. The double monochromators provide a narrow spectral

bandwidth at the selected wavelengths while suppressing stray light at other wavelengths to 1 part in 100,000. This system has a focusing sleeve at the exit portal which would permit the beam being focused on the quartz rod which has been used to date for transillumination using only white light. The main problem with white light is the inability to selectively build-up the contrast of the optical system. The use of monochromatic light permits the selection of wavelengths of light that are absorbed by specific tissue and cellular components. This differential absorption of light by these structures enhances their contrast with the surrounding structures and aids in their visual recognition. When such differences of absorption are sensed by the television tube and converted into an electronic image, the contrast between tissue and cellular components can be enhanced further by adjustment of the brightness and contrast controls on the video monitor. For example, patterns of blood flow can be followed more easily than by using white light by selecting a wavelength of light that is absorbed maximally by hemoglobin in red blood cells (414 mμ). This system will allow more critical observations of the linear velocity of blood flow through the microvascular system as well as passage of these cells through the endothelium of these vessels.

2. Optical Equipment for Panphot Microscope:

At the present time in my laboratory, a fused quartz rod is being used as a light source. The use of a quartz rod (coupled with a monochromatic system) as a transilluminatory light source can provide an adequate amount of light for transillumination of thin tissues such as the fetal or adult mesentery in the rabbit. However, the investigator is somewhat limited in respect to the tissues or organs selected for study since relatively low magnifications are used. Thicker tissues or organs require higher intensities of light in order to

transilluminate through them. This entails the use of a condenser in the optical system. I have in my laboratory a used Leitz Panphot binocular microscope, without optics. This microscope can be adapted for vital microscopy which then can be used for direct in vivo observations of tissues or organs using transmitted or reflected light or alternatively, television microscopy as mentioned in one above. Thus, the part of the experimental protocol which requires examination of organs such as liver in rats can be accomplished. (I have included a reprint, Microvas. Res. 3: 354-360, 1971, which will illustrate the methodology used for television microscopy and how it can be used to study living organs and tissues in situ).

3. Low Light Level Television Camera:

As mentioned in two above, when transilluminating through thick tissues or organs using either a quartz rod or a focusing condenser on a microscope, the amount of light passing through the specimen is greatly reduced from that which would pass through a 10μ thick histologic slide. Thus, the conservation of light becomes imperative. To help offset this loss of light, a higher intensity light source can be used in conjunction with a television system which can provide useful pictures under compromised lighting conditions. The low light level Cohu television camera (2850 series) containing a silicon diode-array vidicon can be used in such conditions. The automatic light range controls are fully operational for scene brightness changes from 0.5 footlambert to 25,000 footlamberts with an f1.4 lens. After seeing a demonstration of this camera, I am convinced of its applicability to television microscopy under compromised lighting conditions and of its superiority over the two vidicon cameras I presently have in my laboratory.

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

urrent

Title of Project	Source	Amount	Duration
Response of the fetal mesenteric microvascular system to maternal carbon monoxide exposure	Louisiana Heart Association	7,300.00	July, 1972-June, 1973
In vivo model for testing effects of pulp capping agents on dental pulp	Institutional Grant	900.00	March, 1973-February, 1974

'ending

(None)

LOUISIANA STATE UNIVERSITY MEDICAL CENTER
1100 FLORIDA AVENUE • NEW ORLEANS, LOUISIANA • 70119

DEPARTMENT OF ANATOMY

June 19, 1973

Frederic W. Nordsiek, Ph.D.
Associate Scientific Director
The Council for Tobacco Research- U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Re: Your Grant Application #912

Dear Dr. Nordsiek:

Please accept my apologies for the omission of the signature of our Comptroller from my grant application. Enclosed is a xeroxed copy of the budget page (p. 28) with Mr. Pohlig's signature.

My delay in replying to your letter was due to my visitation to several laboratories in Scandanavia during May and part of June. While visiting the Department of Experimental Medicine at Pharmacia, AB, in Uppsala, Sweden, I learned a fluorescent technique to study the microcirculation which they have been using for a couple of years. After learning this methodology, I'm convinced of its applicability in studying the permeability of blood vessels under normal or pathologic conditions. In the research grant submitted to the Council for Tobacco Research, I stated that the results from previous experiments in my laboratory on the effects of carbon monoxide (CO) on the fetal or adult mesenteric microvascular system suggest that CO alters the permeability of capillaries or post-capillary venules. The methodology used to study these changes in permeability can easily follow any alteration in the behavior of the cellular elements of blood; however, it is more difficult to document in vivo the passage of plasma constituents across the endothelium which probably precedes any cellular passage due to the effects of CO. This fluorescent technique can be used to study any increased endothelial permeability to plasma which will provide additional information to the changes which have been described in the behavior of blood cells after CO exposure.

In order to examine the effects of CO on the permeability of the fetal and adult microvascular system to plasma constituents, fluorescein conjugated dextran (M. W. 145,000) can be administered I.V. in a 5% Ringer's solution (200 mg./kg.) which is iso-oncotic. The presence of the

labeled dextran within the microvascular system can be examined in vivo by transilluminating the adult or fetal mesenteries with monochromatic light at a wavelenght of 487 mμ and by using a Leitz barrier filter of 515 or 530. Alternatively, selective exciter filters (Leitz BG 12 or KP500) may be used in conjunction with proper barrier filters. Thus, the permeability of the microvascular system to the plasma containing dextran particles can be studied microscopically before and after exposure of the animal to CO. This, then, can be correlated with any increased permeability of cellular elements in the fetal or adult microvascular system. The dextran infusions can be used in any of the experimental groups using rabbits; however, it cannot be used in those groups using rats since rats are hypersensitive to dextran.

I have included a brief description of the technique in this letter in the hope that it may appended to the "methodology" section of the research grant since this technique can document in vivo the passage of plasma across the endothelium of the microvascular system. Furthermore, this technique is far superior to other plasma labeling techniques such as Evan's blue which have been employed in the past to study permeability of vascular endothelium. This in vivo technique will permit a more accurate description of the permeability of small vessels to plasma and will provide a better means to compare the results of this study to those conducted by others who have also similarly described effects of CO on endothelial permeability using other techniques such as electron microscopy.

This technique will not require any additional expenses in the budget. Furthermore, Pharmacia has provided me with a quantity of fluorescein conjugated dextran for use in my laboratory.

Thank you.

Sincerely,

Sam G. McClugage, Jr., Ph.D.
Assistant Professor

SGMc:sar

Enclosure

MICROVASCULAR RESEARCH, 3, 354–360 (1971)

"In Vivo" Microscopic Study of the Response of the Hepatic Microvascular System to Carbon Tetrachloride Poisoning[1]

SAMUEL G. MCCLUGAGE, JR,[2] AND ROBERT S. MCCUSKEY[3]

Department of Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio 45219

Received March 15, 1971

The initial effect of carbon tetrachloride poisoning on the microvascular system and parenchyma of the rat liver was studied using an *in vivo* microscopic method. The results suggest that the initial lesion is in the microvascular compartment; this reaction institutes an inflammatory response characterized by adhesion of white blood cells to the endothelium of sinusoids and central venules, and subsequent diapedesis of white blood cells. Carbon tetrachloride later induces alterations in the parenchyma resulting in fatty changes, hydropic degeneration, and necrosis in a stepwise manner. The combined events, microvascular and parenchymal, produce marked alterations of hepatic blood flow thus promoting anoxia and pathologic lesions.

INTRODUCTION

The pathogenesis of carbon tetrachloride-induced cirrhosis is in some dispute. Aterman (1) described cirrhosis induced by carbon tetrachloride as a chronic inflammatory process that produces alterations in the vascular and parenchymal components of the liver. Contributing features cited have included fatty changes, necrosis, and congested sinusoids (10–12). Petrelli and Stenger have suggested that the wall of the sinusoid might be the initial site of damage by carbon tetrachloride (9). On the other hand, Hase (3), using silicone rubber perfusions, studied the effects of carbon tetrachloride on the "microcirculation" (3) of livers in rats. He concluded that the microvascular lesions always followed the parenchymal lesions. Other investigators (14, 15), studying liver microscopically, reported the response of the exposed, intact liver to this hepatotoxin. Only limited observations could be made in these *in vivo* studies (14, 15) since relatively low magnifications were used with resulting poor resolution, thus prohibiting accurate evaluation of cellular detail. No work has been reported using *in vivo* microscopic methods that permit observations of cellular detail at the limit of resolution of the light microscope (4,5). Thus, the present study was designed to examine concomitantly the hepatic microvasculature and parenchyma in the living state in order to elucidate further the events that antecede cirrhosis.

[1] Presented in part in motion picture form at the Midwestern Association of Anatomists Meeting, Omaha, Nebraska, November 15, 1969.

[2] Present address. Department of Anatomy, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112.

[3] Recipient of N.I.H. Research Career Development Award, AM-42,370.

MATERIALS AND METHODS

One-hundred and fifty male Sprague–Dawley rats (100–125 g) were used. Rats in groups of 25 were injected intraperitoneally or subcutaneously with 0.15 cc CCl_4 (carbon tetrachloride) mixed with 0.15 cc of mineral oil every other day for a period of 2 weeks. Control animals were given placebos of mineral oil or of Ringer's solution. The animals were fed a standard laboratory diet and were given water *ad libitum* throughout the course of the experiment.

An *in vivo* method reported by McCuskey (4,5) was used to study the liver. Animals were anesthetized by intraperitoneal injection of 20% ethyl carbamate (Urethan, 1.5 g/kg). After laparotomy a lobe of the liver was exteriorized by floating it onto a window of Saran Wrap (Dow Chemical, Midland, Michigan) which overlayed a substage condenser. Homeostasis was maintained by irrigating the surface of the liver with Ringer's solution warmed to body temperature (4,5). Transillumination of the exposed edge of the liver was accomplished with monochromatic light (390–650 mμ) brought to the liver by the substage condenser of a modified Leitz Panphot microscope. Observations were made by direct microscopy at magnifications of 100–1000× using Leitz water-immersion objectives (10, 22, 50, and 80 ×) with appropriate oculars, or the optical images were projected onto the photocathode of a RCA vidicon (PK-301) or image orthicon television system (TK-31A) and kinérecorded with a modified Arriflex-16S, 16-mm motion picture camera. Kodak 16-mm Tri-X reversal film was used (4,5). During the 2-week treatment period, the liver was examined immediately after CCl_4 administration and at intervals up to 2 weeks.

Routine histological frozen and paraffin sections were prepared from the liver from some of the animals in order to correlate the *in vivo* microscopic observations with fixed tissue sections; these were stained with oil red O or hematoxylin and eosin.

RESULTS

The structure of the liver was altered progressively during the 2-week period of treatment with CCl_4. Treatment and observations could not be extended past 2 weeks since the CCl_4 induced widespread necrosis, shrinkage, and thickening of the liver preventing adequate definition of the histology of the organ *in situ*.

Carbon tetrachloride had its first visible effect on the microvasculature within 2 hr. This effect became progressively more severe during the first 2 days of treatment. In the majority of the livers observed during this period, the endothelium of the sinusoids became thickened and white blood cells adhered to the walls of the sinusoids in the centrolobular portions of the lobules (Fig. 1). Small aggregates of leukocytes also were observed to adhere to the endothelial wall of central venules. In the healthy animal with optimal circulation, white blood cells were never observed to adhere to the endothelium in this manner (Fig. 2). this diffuse sticking and aggregation resulted in stasis and congestion in the sinusoids, and led to an apparent reduction in the linear velocity of blood flow in the central venules as compared with observations made in control animals. Many of these white cells also passed through the endothelium of the central venules and sinusoids and entered the extravascular space. Subsequently, in many

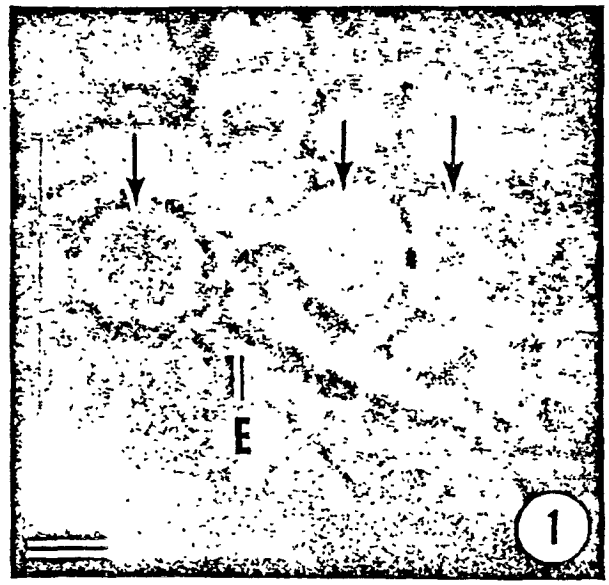

FIG. 1. Aggregation and adhesion of white blood cells (arrows) to endothelium (E) of sinusoid 2 hr after administration of CCl_4. Single frame from motion picture. Size marker is 5 μ.

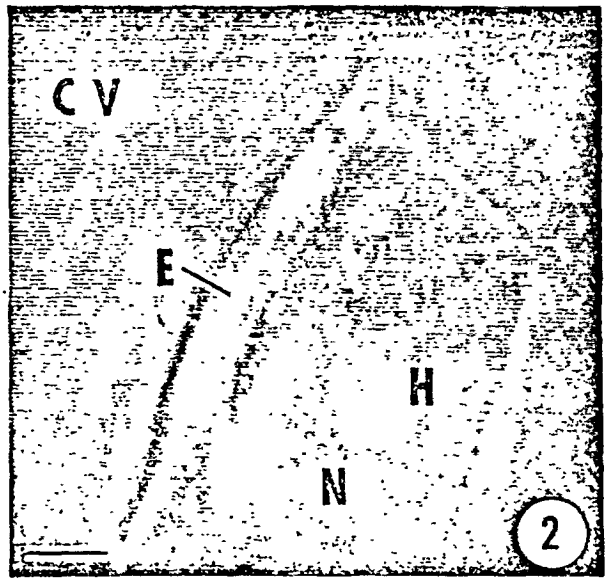

FIG. 2. Central venule (CV) with optimal circulation in nontreated, healthy liver. Note that there are no white blood cells adhering to the endothelium (E); N, nucleus of hepatic cell (H). Single frame from motion picture. Size marker is 5 μ.

lobules red blood cells extravasated through the endothelium of the sinusoids and central venules resulting in minute hemorrhages. Toward the end of the treatment period, central venules were obscured by centrolobular necrosis, hemorrhage, and moderate fibrosis.

While these changes produced marked alteration of blood flow, not all vascular channels were affected; some vessels and lobules were relatively normal in appearance. The lesions described were always most severe in the centrolobular areas while the portal areas of the same lobules were normal in appearance, exhibiting little vascular involvement.

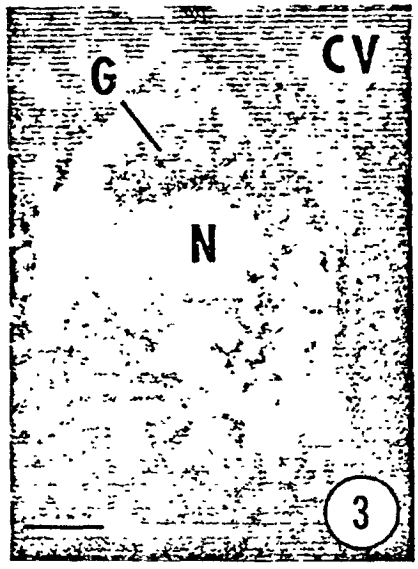

FIG. 3. Hepatocytes with perinuclear granulation (G), N, nucleus; CV, central venule. Five days after initial administration of CCl_4. Single frame from motion picture. Size marker is 5 μ.

Visible parenchymal alterations followed the microvascular response. During the first 4 days of exposure to CCl_4, small fat vacuoles developed in hepatocytes extending from the midlobular region to the central venules. These vacuoles displaced the nuclei to the periphery of the cell. Cells in the periportal areas exhibited no detectable fatty change.

During the intermediate stage of treatment, 5–9 days, fat accumulation became more severe but remained predominantly in the centrolobular region. Cells now contained multiple vacuoles; others exhibited perinuclear granulation, especially cells adjacent to central venules (Fig. 3). Fibrosis became evident in a few necrotic centrolobular areas.

In the later stages, 10–14 days, in addition to the above changes, large hypertrophied hepatocytes were evident in centrolobular and midlobular areas. The hepatocytes contained a mass of perinuclear granules surrounded by a homogeneous cytoplasmic matrix; this effect was a progressive development from the parenchymal changes

described in the intermediate stage. At this time hepatocytes in the periportal areas of many lobules had undergone some fatty change while the centrolobular areas of these same lobules were necrotic.

Throughout the course of the experiment, increasing deposits of fat, debris, and hemorrhage beneath the capsule made visualization difficult and at times impossible.

Frozen and paraffin sections confirmed the lesions observed *in vivo* in the microvascular and parenchymal compartments.

DISCUSSION

The hepatotoxicity of CCl_4 has been the subject of many investigations. The variability of results may be attributed to the different dosages of CCl_4 used, the different routes of administration, and the age and sex of the animal since all of these factors are known to affect the hepatotoxicity of CCl_4 (13). By using low doses of CCl_4 in conjunction with a short time sequence (2 weeks), it was possible to study in the living state some of the initial effects of CCl_4 upon the rat liver. The histologic alterations induced by CCl_4 and observed sequentially *in vivo* were the following: (a) endothelial damage with adhesion of white cells to the walls of sinusoids and central venules; (b) reduced blood flow through sinusoids and central venules as compared with control animals due to plugging of these vessels by adherent white blood cell masses; (c) diapedesis of white cells into the extravascular compartment; (d) fatty metamorphosis and hydropic degeneration of parenchymal cells, centrolobular necrosis, and hemorrhage; (e) further reduction of blood flow through sinusoids as a result of impingement of hypertrophied hepatocytes on the sinusoids; and (f) widespread vascular congestion and necrosis. These results suggested that the primary site of injury of the CCl_4-poisoned rat liver might be in the microvascular system.

The alterations described above are not unexpected since CCl_4-induced cirrhosis has been classified as a chronic inflammatory condition (1). The results of this study are in agreement with the findings of Zweifach *et al.* (16) who studied how damaged endothelium alters the behavior of white cells during an inflammatory response. The adhesion and aggregation of white blood cells to endothelium may be a cause of tissue anoxia by greatly reducing blood flow through the microvascular system of the liver. Plugging of sinusoids and central venules by leukocytes, diapedesis of white blood cells, and hemorrhage reflect endothelial damage (16). Apparently this damage is initially induced by the CCl_4, and later augmented by anoxia due to a decreased blood flow through the sinusoids. Petrelli and Stenger (9) were able to reduce the hepatotoxic effects of CCl_4 by giving trypan blue prior to the CCl_4. The trypan blue increased the cytoplasmic mass of the lining cells of the sinusoid, thus reducing the accessibility of the extravascular compartment to CCl_4. This suggests, as does this report, that the initial lesion is in the microvascular compartment. In addition, subsequent swelling of parenchymal cells further reduced blood flow. Since the centrolobular areas are farthest from the oxygenated blood supply, they would be affected first by the anoxia (4,6); the most severe parenchymal lesion was always in the centrolobular region.

Rice *et al.* (10) thought that the initial lesion in CCl_4 hepatotoxicity was not vascular. They believed that blood flow played a minor role in furthering the damage once the parenchymal lesion had been induced. However, an increasing biphasic resistance to

flow in perfused livers, described by Rice and Plaa (11), could well have been caused by vascular lesions such as we observed rather than by obstruction from increased cellular triglyceride content in the early phase or later necrosis. Nakata and Higaki (7) reported that the hemodynamic changes always followed the appearance of parenchymal lesions in their perfused liver preparations. It would be of considerable interest to determine if the intravascular hemodynamic changes observed *in vivo*, i.e., adhesion and aggregation of white blood cells, can be induced in an organ which is perfused. Although the vascular lesions described in this report were not observed in other *in vivo* studies (14,15), this may be due to differences in methodology, especially the use of high magnifications in this work as opposed to low magnifications and poor resolution.

Although the process of fixation probably washed out many of the adherent white blood cells, tissue sections from this experiment did suggest areas of white cell adhesion and diapedesis through the walls of sinusoids and central venules. Some of the white cells within the vessels were enmeshed in a fibrin matrix.

The parenchymal changes observed *in situ* after CCl_4 poisoning follow conditions described by Gall (2) in cases of nutritional cirrhosis, namely, hepatocyte disintegration, focal necrosis, and a variety of cytoplasmic changes. Pseudolobule formation and fibrous interconnections were lacking probably because of the relatively low dosage of CCl_4 and the short treatment period. For the most part, hepatic architecture was maintained, but widespread centrolobular necrosis occurred in the later stages of treatment.

During the 2-week sequence of treatment, the hepatocytes appeared to undergo three progressive stages of morphologic alteration, i.e., fatty change, hydropic degeneration, advanced hydropic degeneration, and necrosis. In any one stage of parenchymal change the most advanced lesions were always nearest central venules. Thus in the final stage of treatment, at 10–14 days, the parenchymal lesions from within the center of the lobule outward to the periphery, mimicked the CCl_4-induced alterations described by other investigators using light microscopy, namely, necrosis, hydropic degeneration, and fatty changes. The most severe change was always farthest from the oxygenated blood supply (4,6)

The distribution of lesions observed *in vivo* as areas of fatty change, hydropic degeneration (balloon cells) (8), and necrosis was confirmed by the use of frozen tissue sections stained with oil red O.

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2. GALL E. A. (1960). Posthepatitic, postnecrotic, and nutritional cirrhosis. A pathologic analysis. *Amer. J. Pathol.* 36, 241.
3. HASE, T. (1966). Hepatic microcirculatory changes in acute and chronic carbon tetrachloride poisoning in rats. *Amer. J. Pathol.* 49, 1069.
4. MCCUSKEY, R. S. (1966). A dynamic and static study of hepatic arterioles and hepatic sphincters. *Amer. J. Anat.* 119, 455.
5. MCCUSKEY, R. S. (1968). Dynamic microscopic anatomy of the fetal liver. III. Erythropoiesis. *Anat. Rec.* 161, 267.
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7. NAKATA, K., AND HIGAKI, K (1969). Relationship between circulatory disturbance and histological lesions in the isolated rat liver resulting from CCl_4 poisoning. *Microvasc. Res.* 1, 379.

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Response of the Fetal Mesenteric Microvascular System to Maternal Hypoxia[1] (34277)

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Several studies have been reported on general cardiovascular responses of the fetus to maternal hypoxia or anoxia. These were reviewed recently by Dawes (1) and Rudolph and Heymann (2). The specific response of the fetal microvascular system to maternal hypoxia, however, has not been reported due, in part, to the difficulty involved in examining these vessels directly *in vivo* with the light microscope while maintaining homeostasis. This poor understanding of the microvascular system has prompted a series of studies of these vessels *in vivo* in rabbit fetuses with their placental circulations intact (3–6). The present paper reports the effect of maternal hypoxia on the fetal mesenteric microvascular system.

Materials and Methods. The mesenteries of 50 fetal and 15 adult pregnant rabbits (New Zealand albino) were studied. Fetal preparations and adult preparations were studied independently since technical complications did not permit simultaneous microscopic observations of fetal and adult mesenteries. In both preparations pregnant rabbits were anesthetized with ethyl carbamate (Urethane, 1.5 g/kg). To study the fetal mesentery a fetus was exteriorized with its placental circulation intact on various days of gestation between days 25 and 32 (av gestation in the rabbit is 32 days) and the fetal mesentery was exposed surgically. Homeostasis was maintained by constant irrigation with Ringer's solution of the surface of the mesentery as well as the fetal body surface which was covered with gauze sponges. The temperature of the Ringer's was maintained at the maternal body temperature by regulating heaters (3–6). In addition, the ambient air surrounding the fetus was maintained at 37.5° by a Sage "air curtain" with its controlling thermister probe placed on the surface of the fetus. To study the mesentery of the pregnant adult rabbit, the uterus was displaced and a loop of bowel was exposed. Homeostasis was maintained as in the fetus.

Observations of the mesentery of the fetus or of the pregnant adult were accomplished by transillumination of the tissue with light conducted to the mesentery by a hollow, fused quartz-rod (7) and examination with a Leitz stereo-binocular microscope equipped with 2×, 4×, 8×, and 12× objectives and 12.5× and 18× oculars. Using these optics magnifications of 25–216× were obtained. Alternatively, a modified Leitz compound monocular microscope was used equipped with 10×, 22×, 50×, and 90× water immersion objectives and a 10× ocular to provide magnifications to 900×. Measurements of the internal diameters of vessels were secured with a calibrated micrometer disc in the oculars.

To study the response to hypoxia of the mesenteric microvasculature of the fetus and pregnant adult, the mother received a mixture of 8% O_2/92% N_2 gas for 30 min by means of a closed circuit anesthetic machine. Then the low oxygen mixture was removed and the animal was allowed to recover breathing room air. This procedure also was repeated in fetuses and mothers to whose mesenteries an alpha-adrenergic blocking agent, phentolamine (50 μg), or a beta-adrenergic blocking agent, propranolol (50 μg), had been applied topically. Maternal

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and fetal blood pH, pO_2 and pCO_2 were monitored using an ultramicro blood gas analyzer (Instrumentation Laboratories, model 123-S11, 125A).

The effect of catecholamines on the mesenteric microvasculature of the fetus and adult was tested by local, topical application of epinephrine (10 μg), and norepinephrine (10 μg) both before and after application of phentolamine or propranolol.

The responses of the fetal mesenteric microvasculature to hypoxia and subsequent recovery were recorded cinéphotomicrographically and were compared with those in the maternal mesenteric microvasculature.

Results. The responses of the fetal mesenteric microvasculature to acute hypoxia in the mother were vasoconstriction, reduced flow in large arterioles and venules (100–300 μ i.d.), severely reduced flow in small arterioles and venules (less than 100 μ i.d.), and elimination of flow in most capillaries. These responses occurred within 30 min after the administration of 8% O_2 was initiated. Recovery occurred within 20 min after the removal of the low oxygen mixture (Fig. 1). Similar responses were observed in the maternal mesenteric microvasculature but the responses were seen within 20 min with a lag in the initiation of the vasoconstriction and with recovery within 5–10 min (Fig. 1). During recovery vessel diameter was restored in parallel with blood pO_2 while pCO_2 still was elevated and pH depressed.

The above responses could be mimicked by local, topical application of epinephrine or norepinephrine. Local topical application of phentolamine blocked the above vasoconstrictive responses caused by hypoxia (Fig. 1), epinepherine, or norepinephrine in both the fetal and maternal vessels, and occasionally resulted in a slight dilatation of these vessels. Propranolol, however, failed to block the vasoconstrictive response to hypoxia. Acute maternal hypoxia did not induce tissue edema nor did it lead to intravascular erythrocyte aggregation and sludging in the vessels examined.

Discussion. These data illustrate that the response of the fetal mesenteric microvascular system to hypoxia is vasoconstriction and suggests that this response is mediated by an oxygen dependant, alpha-adrenergic mechanism since: (i) the response could be mimicked by the administration of epinephrine or norepinephrine and could be blocked by an alpha-adrenergic blocking agent, phentolamine, but could not be blocked by a beta-adrenergic blocking agent, propranolol; and (ii) vessel diameter returned in parallel with blood pO_2 while blood pCO_2 remained elevated and blood pH depressed. Thus, it would seem that recovery following hypoxia in the fetal microvasculature and reestablishment of blood flow through capillaries of the mesenteric tissue is not so much dependant on the blood acid–base balance and pCO_2 as it is upon blood pO_2, a finding that is in agreement with the results of Godfrey (8).

At this time, however, it is not clear whether the vasoconstriction is due to reflex neural mechanisms initiated by chemoreceptors, is due to humoral mechanisms, e.g., elaboration of epinephrine from the suprarenal, or is possibly a direct effect of hypoxia on the vessel wall. While the existence of functional chemoreceptors and autonomic innervation in the fetus is not clear (1, 2, 9–13), several studies suggest the importance of catecholamine release from the suprarenal during the last half of gestation in the response of the fetus to stress (1, 2, 14). Unfortunately, there is little or no information concerning the sensitivity of the fetal systemic vascular wall to varying concentrations of oxygen in the blood. Studies on isolated adult vessels, however, indicate that hypoxia induces vasodilatation, except in the lung where vasoconstriction is the result (15). While vasoconstriction of pulmonary vessels in response to hypoxia also has been demonstrated to be a direct, local effect in the fetus (1), there is little information concerning such direct action in the fetal systemic vessels. In addition, the relative sensitivities of the fetal systemic vessels compared with the adult to varied oxygen concentrations have not been reported.

In this study the data suggest that the response of the maternal and fetal vessels to maternal hypoxia are equivalent. Both maternal and fetal arterioles constricted approx-

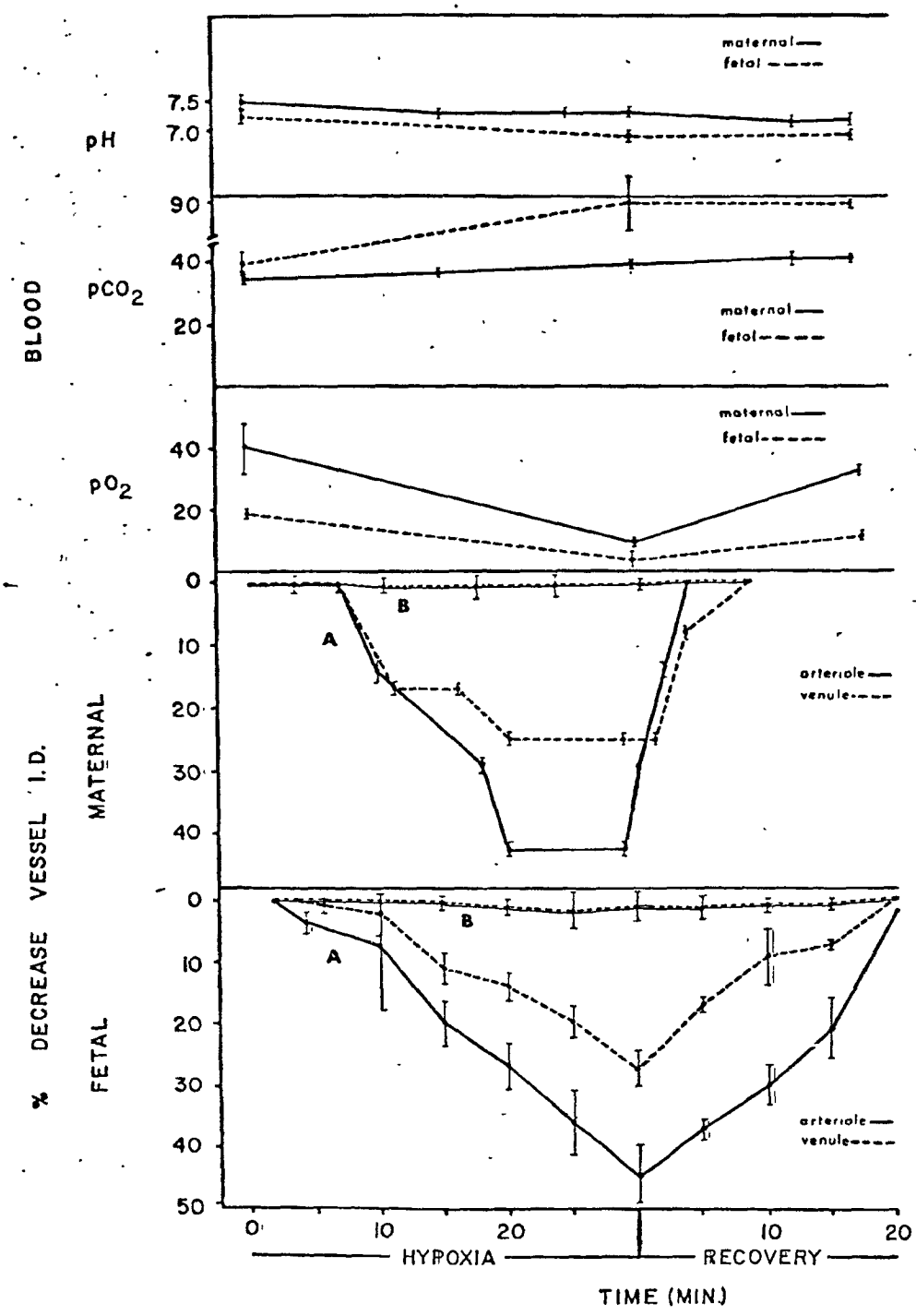

FIG. 1. Changes (± the standard error of the mean) in the internal diameters of vessels in fetal and maternal mesenteric microvasculature, and changes in pH, pO_2, and pCO_2 of the fetal and maternal blood, during hypoxia and recovery in anesthetized rabbits: A, normal response to hypoxia and following administration of propranolol; B, response to hypoxia after administration of phentolamine.

imately 45% while venules constricted approximately 25%. The smaller degree of venular constriction suggests that these vessels may have less functional innervation, may be less sensitive to catcholamines released from the suprarenal, or may be less sensitive to low oxygen saturation of the blood. The most probable explanation, however, is that these vessels contain considerably less smooth muscle in their walls than do their companion arterioles and are less capable of vasoconstriction.

Summary. The effect of maternal hypoxia on the microvascular system of fetal and pregnant adult rabbits was studied. The response of the fetal mesenteric microvasculature to hypoxia was vasoconstriction, reduced flow in the large arterioles and venules, and severely reduced flow in most capillaries. This response occurred within 30 min after initiation of 8% O_2; recovery occurred within 20 min after removal of the low oxygen mixture. Similar findings were obtained in the maternal mesenteric microvasculature but the responses were more rapid, occurring within 20 min, with recovery within 5–10 min. The responses appeared to be mediated by an oxygen dependant, alpha-adrenergic mechanism since, during recovery, flow and vascular diameter were restored in parallel with the blood pO_2 even though blood pH still was depressed and pCO_2 was elevated, and since the vasoconstrictive response could be blocked by phentolamine but not by propranolol.

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